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Analysis of promoter regions for the spinach chloroplast *rbc*L, *atp*B and *psb*A genes

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A promoter-deletion derivative of the spinach *trn*M2 gene was used for the identification and characterization of the promoter regions for the spinach chloroplast RuBisCo large subunit (*rbc*L), ATPase β-subunit (*atp*B) and Q_B-polypeptide (*psb*A) genes. The DNA sequences 5′ upstream from the transcriptional start sites of these genes share homology with the ctp1 and ctp2 arrangement found for the *trn*M2 transcription unit and the canonical *Escherichia coli* '−10' and '−35' promoter regions. Synthetic DNA fragments of ~40-bp regions, including the defined transcriptional start sites and proximal residues, from *rbc*L, *atp*B and *psb*A, were fused to the *trn*M2 deletion mutant 51. The promoter-fusion constructs direct the correct transcription of $tRNA_2^{Met}$ in the chloroplast extract with distinct efficiencies. The ctp1- and ctp2-like elements in the *trn*M2, *rbc*L and *psb*A promoter regions can be interchanged to yield functional chimeric promoters of varying strengths. As a result, ctp1 sequences from *atp*B and *psb*A, *trn*M2 and *rbc*L, respectively, can be ordered TTGACA >TTGCTT>TTGCGC with respect to their intrinsic strengths. Single base pair changes were introduced into the ctp2-like element in the *psb*A promoter region. In analogy to similar base pair changes which lower promoter efficiency in *E. coli*, these mutations result in reduced transcription levels in the chloroplast extract. The data are consistent with a prokaryotic model for chloroplast promoter function.

Key words: chloroplast protein genes/promoter structure/chimeric promoter/*in vitro* mutagenesis/*in vitro* transcription

Introduction

The chloroplast genomes of higher plants resemble in many aspects the organization of prokaryotic genomes (Bohnert *et al.*, 1982; Whitfeld and Bottomley, 1983). Most striking is the clustering of several protein-coding genes into polycistronic transcription units and the homology in 5′ upstream regions of several genes with prokaryotic promoter sequences (Krebbers *et al.*, 1982; Zurawski *et al.*, 1982a; Westhoff *et al.*, 1983, 1985; Whitfeld and Bottomley, 1983; Crouse *et al.*, 1984). As a result *Escherichia coli* RNA polymerase has been used to study enzyme binding and transcription of cloned chloroplast genes (Tohdoh *et al.*, 1981; Zech *et al.*, 1981; Gatenby *et al.*, 1981; Hanley-Bowdoin *et al.*, 1985a). For example, in the gene for the large subunit of ribulose-1,5-bisphosphate carboyxlase (*rbc*L) *E. coli* RNA polymerase initiates transcription at discrete sites and produces transcripts that have 5′ termini identical to those found in the chloroplast (Shinozaki and Sugiura, 1982; Erion *et al.*, 1983). These studies, although suggestive, do not decisively demonstrate that similar promoter sequences are recognized by chloroplast and *E. coli* RNA polymerases. In fact, the two transcriptional systems do have identifiable differences. The chloroplast RNA polymerase activities responsible for the transcription of tRNA and protein coding genes in spinach and *Euglena* are resistant to rifampicin (Gruissem *et al.*, 1983a, 1983b). In contrast, *E. coli* RNA polymerase is unable to transcribe these DNA templates when added to chloroplast extracts in the presence of the antibiotic (Gruissem *et al.*, 1983a). Similar results have been reported for chloroplast RNA polymerases from other plants (Bottomley *et al.*, 1971; Orozco *et al.*, 1985). In addition, not all upstream regions of plastid genes appear to have DNA sequences that resemble the canonical '−10' (TATAAT) and '−35' (TTGACA) regions that are important sequence elements in prokaryotic promoters (Gruissem and Zurawski, 1985a).

In the first report on the physiological significance of prokaryotic-type promoter elements in chloroplast gene transcription the spinach *trn*M2 locus (encoding $tRNA_2^{Met}$) was used as model gene in a homologous *in vitro* transcription system (Gruissem and Zurawski, 1985b). The *trn*M2 promoter region has two sequence elements (ctp1, TTGCTT and ctp2, TATAAT) that are homologous in sequence and relative location to the prokaryotic '−35' and '−10' sequences. Deletion analysis demonstrated that sequences distal to ctp1 are not required for transcription. Furthermore, oligonucleotide-directed mutagenesis confirmed that both ctp1 and ctp2, in a fixed relative location, were required for transcription and that sequences between ctp1 and ctp2 are relatively unimportant. These results are entirely consistent with the applicability of the prokaryotic promoter model to the *trn*M2 promoter.

We have extended the analysis of chloroplast promoter regions to protein-coding genes in higher plants. The 5′ DNA sequences upstream from the spinach chloroplast genes for the large subunit of the ribulose-1,5-bisphosphate carboxylase (*rbc*L), the β-subunit of the ATP synthetase (*atp*B), and the 32-kd polypeptide of photosystem II (*psb*A) share homology with the ctp1 and ctp2 arrangement found for the *trn*M2 transcription unit (Zurawski *et al.*, 1981, 1982a, 1982b). In chloroplast genomes of higher plants, the *rbc*L transcription unit and the gene for the β-subunit of ATP synthetase (*atp*B) are adjacent and transcribed divergently from a small intergenic region (Mullet *et al.*, 1985). The gene for the ε-subunit of ATP synthetase (*atp*E) is organized with *atp*B into a polycistronic transcription unit (*atp*BE; Krebbers *et al.*, 1982; Zurawski *et al.*, 1982a; Shinozaki and Sugiura, 1982; Zurawski and Clegg, 1984). The gene for the 32-kd polypeptide of photosystem II (*psb*A) in the spinach chloroplast genome is organized into a polycistronic transcription unit, with the gene for $tRNA_1^{His}$ (*trn*H1) located ~140 bp downstream from the *psb*A coding region (Zurawski *et al.*, 1984; Gruissem *et al.*, 1986). The particular organization of these transcription units may be important for the regulation of their expression.

In the present study we used a promoter-deletion derivative of the *trn*M2 locus as a tool for the identification and characterization of the spinach chloroplast promoter regions for these protein-coding genes. We show that ~40-bp regions, including the

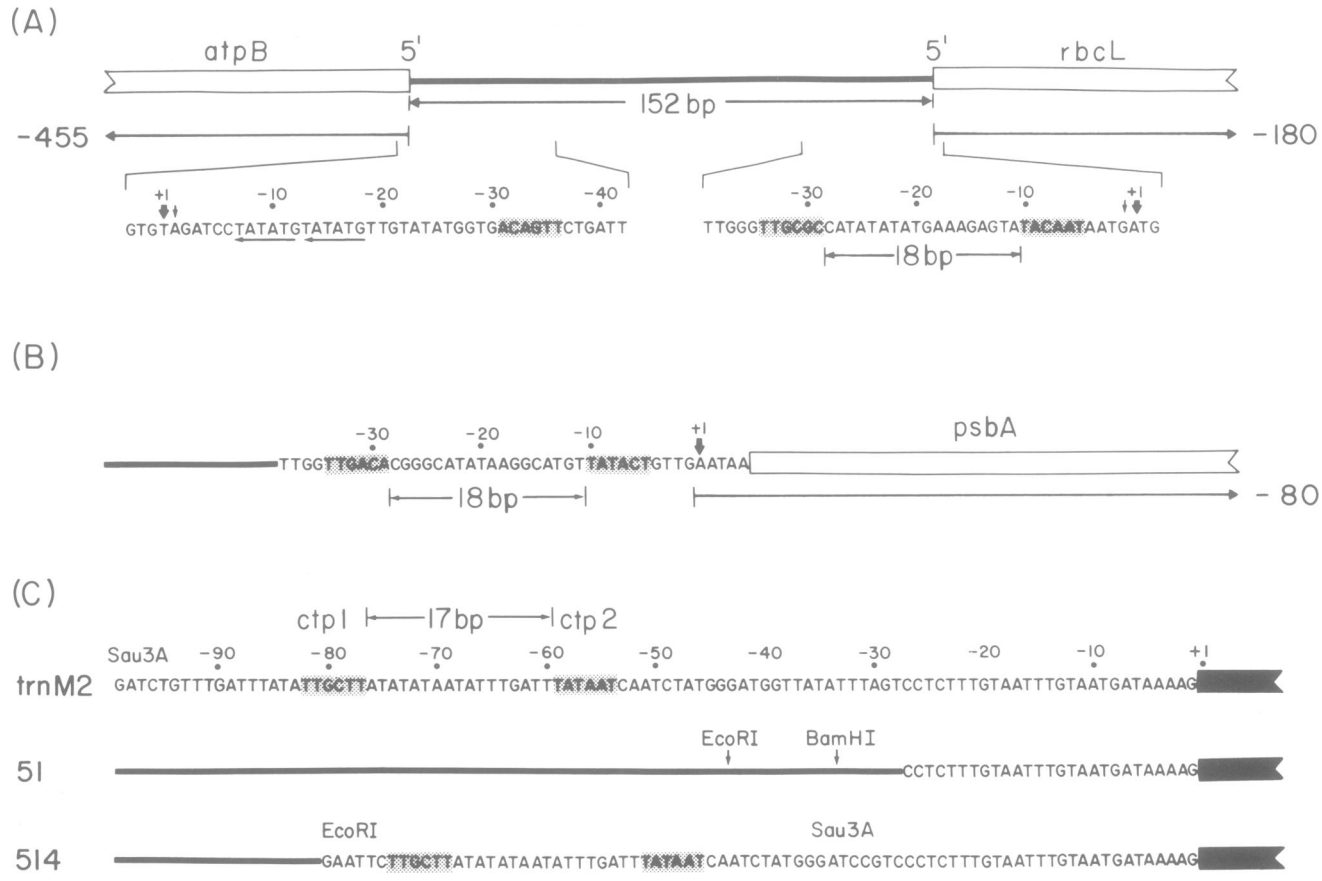

Fig. 1. Schematic diagram and DNA sequences of 5′ upstream regions for spinach plastid genes used for the construction of *trn*M2/promoter fusion templates. The regions surrounding the transcription initiation sites are shown for the '−180' trancript of *rbc*L (Zurawski *et al.*, 1981) and the '−455' *atp*B transcript (Zurawski *et al.*, 1982a) from the *rbcL/atp*B intergenic region in **(A)**, and the '−80' transcript for *psb*A (Zurawski *et al.*, 1982b) from the *trn*K1/*psb*A intergenic region in **(B)**. The open boxes represent transcribed, but untranslated regions for *rbc*L, *atp*B and *psb*A. Major and minor transcription initiation sites relative to their respective open reading frames and the direction of transcripts are indicated by arrows. The bars in **(A)** and **(B)** represent 5′ upstream regions. The shaded boxes in the DNA sequences from the 5′ upstream regions of *rbc*L, *atp*B and *psb*A designate conserved sequences with homology to prokaryotic consensus promoter sequences (Hawley and McClure, 1983) and promoter sequences from the spinach plastid *trn*M2 gene (Gruissem and Zurawski, 1985b). Arrows underline DNA sequences for the *atp*B gene from −18 to −7 which have the structure of a direct repeat. The spacing of conserved DNA sequences is indicated for *rbc*L and *psb*A. **(C)** shows the 5′ upstream DNA sequence of *trn*M2 with ctp1 and ctp2 (shaded sequence), which have been identified as promoter elements (Gruissem and Zurawski, 1985a). 51 is a *trn*M2 5′ deletion mutant which has been used for construction of *trn*M2/promoter fusion templates as described in Materials and methods. *Eco*RI and *Bam*HI indicate restriction enzyme sites in the polylinker region of pdX11, which are relevant to the construction. 514 is derived from 51 by fusion of *trn*M2 DNA sequences from −41 to −82 to this mutant. The transcription properties of these constructs have been described (Gruissem and Zurawski, 1985b). The filled boxes and bars represent the *trn*M2-coding region and vector DNA, respectively.

defined *in vivo* transcriptional start sites and proximal residues, from *rbc*L, *atp*B and *psb*A, can direct the correct transcription of $tRNA_2^{Met}$ when fused to the *trn*M2 promoter mutant. We also determine that the ctp1- and ctp2-like elements in chloroplast promoters can be interchanged to yield functional promoters of varying strengths. Again, these results are entirely consistent with rules that have been established for prokaryotic promoters. In a preliminary survey of one chloroplast promoter region, we show that single base pair changes that would by analogy be promoter-down in the prokaryotic promoter also result in reduced transcription levels in the chloroplast extract.

Results

The spinach chloroplast rbcL, atpBE and psbA-trnH1 transcriptional units

rbcL. In higher plants *rbc*L is a monocistronic transcriptional unit and is located in the large single copy region of the chloroplast genome. In spinach, the 5′ end of the *rbc*L transcribed region is 152 bp distal to the 5′ end of the *atp*B gene, thus forming a relatively short intergenic region from which both genes are transcribed divergently (Orozco *et al.*, 1985; Mullet *et al.*, 1985; Figure 1A). The complete nucleotide sequences for both genes have been determined and the 5′ ends of their *in vivo* and *in vitro* transcripts have been mapped (Zurawski *et al.*, 1981; Orozco *et al.*, 1985; Mullet *et al.*, 1985). Multiple transcripts are observed for both genes *in vivo*, but only the '−180' and '−455' mRNAs from *rbc*L and *atp*B, respectively, have been shown by *in vitro* capping analysis to be the primary transcripts (Hanley-Bowdoin *et al.*, 1985b). The primary *rbc*L and *atp*B transcripts have 5′ termini 178 − 179 and 453 − 454 nucleotides upstream from their respective protein-coding regions. *In vitro* transcription experiments using *rbc*L and *atp*B templates support the conclusion that the smaller RNAs from these genes are processing products of the primary transcripts (Hanley-Bowdoin *et al.*, 1985b). It is most likely, therefore, that the promoter elements for these genes are located in the intergenic region. Sequences of the *rbc*L-*atp*B intergenic region are available for spinach (Zurawski *et al.*, 1982a), tobacco (Shinozaki and Sugiura, 1982) pea (G.Zurawski, unpublished data), maize (Krebbers *et al.*, 1982) and barley (Zurawski and Clegg, 1984) chloroplast DNAs. The

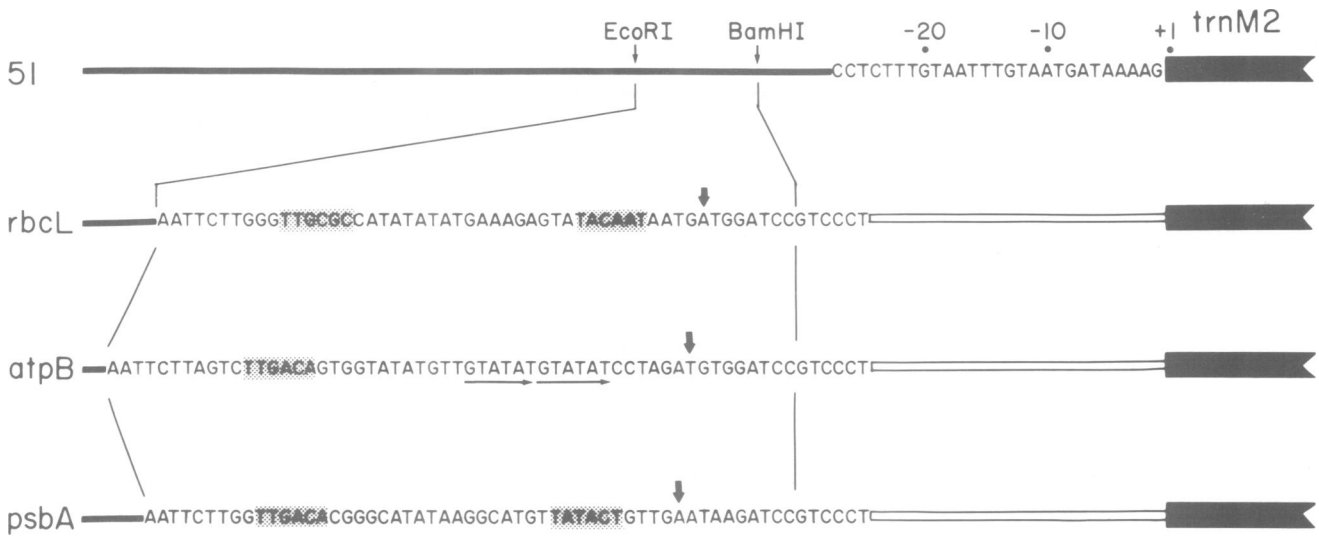

Fig. 2. Construction of *trn*M2/promoter fusion templates. A detailed construction procedure is described in Materials and methods. *rbc*L designates a template in which *rbc*L DNA sequences from +3 to −39 were fused to *trn*M2 deletion mutant 51 to construct 51-*rbc*L. For *atp*B, DNA sequences from +4 to −42 were used in the construction of 51-*atp*B. Similarly, *psb*A designates the template 51-*psb*A, which resulted from fusion of *psb*A DNA sequences from +5 to −38 to deletion mutant 51. The filled boxes and bars represent the *trn*M2-coding region and pdX11 sequences, respectively. The open bar indicates the *trn*M2 5′ upstream DNA sequence from −1 to −27. The sequence from −27 to the respective *Bam*HI cloning sites is from the polylinker region of pdX11.

sequence 39 bp proximal to, and 20 bp distal to, the *rbc*L transcription start site is strongly conserved between the various chloroplast DNAs. This is in marked contrast to the adjacent regions where no sequence homology is observed. The conserved 59-bp region contains the sequence element TTGCGC at −34 that is analogous to the *trn*M2 ctp1 TTGCTT sequence and the prokaryotic '−35' TTGACA sequence. At −10 a sequence, TACAAT, occurs that is analogous to the *trn*M2 and prokaryotic '−10' TATAAT sequences. The spacing between these sequence elements is 18 bp for the *rbc*L, 17 bp for *trn*M2 and 17 ± 1 bp for the canonical prokaryotic promoter. For the above reasons, we selected the *rbc*L sequence from −39 to +2 (Figure 1A) as a likely *rbc*L promoter region. The synthesis, cloning and characterization of this region is described below.

atpBE. The *atp*B-coding region is co-transcribed with the adjacent *atp*E-coding region (Zurawski *et al.*, 1982a). The sequences for 36 bp proximal to, and 18 bp distal to, the spinach chloroplast *atp*BE transcription start site is highly conserved in pea (G. Zurawski, unpublished) and tobacco (Shinozaki and Sugiura, 1982), but not in maize or barley (Krebbers *et al.*, 1982; Zurawski and Clegg, 1984). This conserved sequence contains the sequence element TTGACA at −36 that is homologous to the *trn*M2 ctp1 and the prokaryotic '−35' sequence. This putative promoter region, however, contains no obvious analogue of the ctp2 or the '−10' sequences. We selected the *atp*B sequence from −43 to +4 (Figure 1A) as a likely *atp*BE promoter region. The synthesis, cloning and characterization of this sequence is described below.

psbA-trnH1. The spinach chloroplast gene for the 32-kd thylakoid membrane protein (*psb*A) is located in the large single copy region adjacent to one inverted repeat unit. The *psb*A gene is apparently co-transcribed with the distal gene encoding $tRNA_1^{His}$ (*trn*H1, Zurawski *et al.*, 1984; Gruissem *et al.*, 1986). A single transcriptional start site has been identified for *psb*A transcription in spinach (Zurawski *et al.*, 1982b), tobacco (Zurawski *et al.*, 1982b; Sugita and Sugiura, 1984) and soybean (Spielmann and Stutz, 1983) chloroplast DNAs. The region surrounding this start site is highly conserved and contains the sequence element TTGACA (analogous to ctp1 and the '−35' sequence) at the −34 and the sequence TATACT (analogous to ctp2 and the '−10' sequence) at −10. The spacing between these sequence elements is 18 bp. We selected the *psb*A sequence from −39 to +5 (Figure 1B) for the work described below.

Fusion of heterologous chloroplast promoters to the trnM2 coding region

We have presented evidence that a sequence of 42 nucleotides from a region proximal to the *trn*M2-coding region was critical to *in vitro* promoter function (Gruissem and Zurawski, 1985b). Part of the evidence was that promoter activity of a deletion that removed all but 28 proximal residues (51, Figure 1C) could be restored by reintroducing a synthetically derived oligomer that encoded 44 residues from the putative *trn*M2 promoter region (514, Figure 1C). A further test to delimit chloroplast promoter sequences would be to replace the putative *trn*M2 promoter region with heterologous chloroplast promoters. If ctp1/ctp2-like sequences in the *rbc*L, *atp*B and *psb*A 5′ upstream regions are functional promoter elements, then DNA fragments containing these sequences should direct transcription of $tRNA_2^{Met}$ when fused to *trn*M2 mutants from which the endogenous promoter had been deleted. We therefore selected the three putative promoter regions for the above described genes, synthesized oligomers encoding both strands with overlapping *Eco*RI and *Bam*HI cohesive ends, and cloned the synthetic DNA fragments into *trn*M2 deletion mutant 51. The resulting constructions shown in Figure 2 are analogues of the *trn*M2 514 construct. This approach also allows direct quantitation of *in vitro* $tRNA_2^{Met}$ transcription products and avoids S1 mapping or primer extension techniques, where endogenous RNA potentially contributes to a significant background signal.

Transcription of $tRNA_2^{Met}$ from heterologous promoter regions

The transcriptional properties of the *trn*M2/promoter fusion constructs were analyzed in the chloroplast *in vitro* system. Figure 3A shows that the synthetic DNA fragments from the 5′ upstream regions of *rbc*L, *atp*B and *psb*A all direct the synthesis of $tRNA_2^{Met}$ at levels significantly above (8- to 18-fold) the *trn*M2

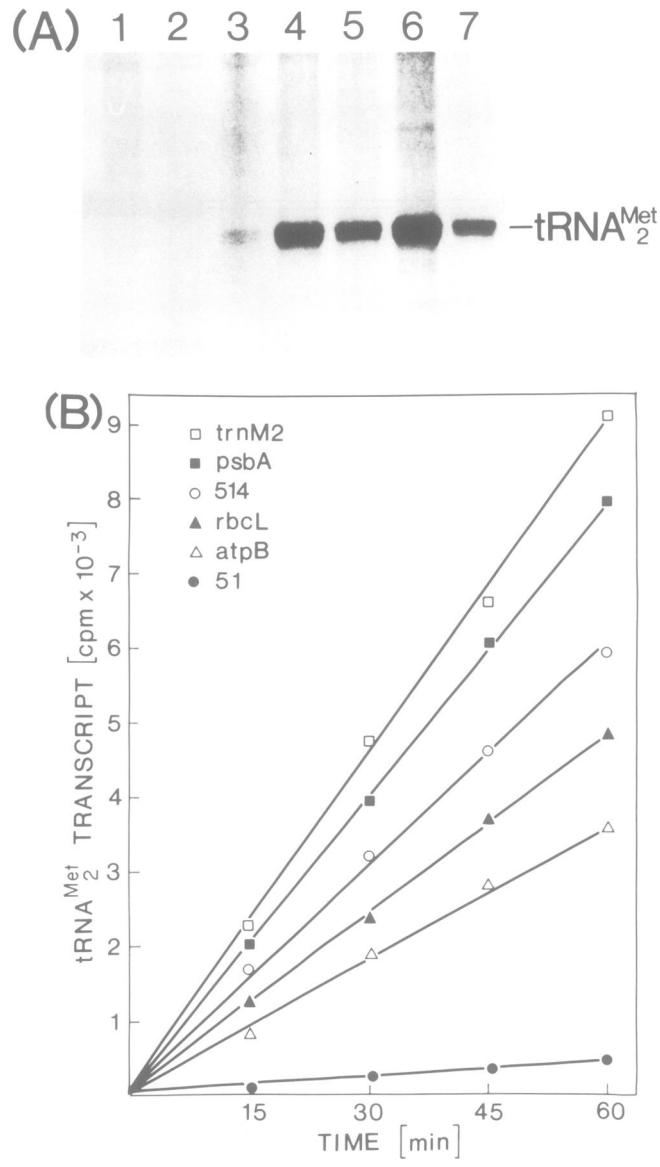

Fig. 3. Correct *in vitro* transcription of tRNA$^{Met}_2$ from *trn*M2/promoter fusion templates. **(A)** Transcription in the spinach chloroplast extract was routinely performed with equal copy numbers for the wild-type gene (*trn*M2 *Sau*3A-*Xba*I fragment in pdX11), the parental plasmid 514, deletion mutants and *trn*M2/promoter fusion constructs in separate reactions. Form I plasmid DNAs (60 μg/ml) were transcribed *in vitro* as described in Materials and methods. The incubation time was 60 min at 25°C. Differences in the relative transcription efficiencies of templates with varying amounts of DNA were only observed in chloroplast transcription extracts from young leaves (1 – 5 cm). After autoradiography with unflashed Kodak XRA5 film, the tRNA$^{Met}_2$ transcription products were excised from the gel and incorporation of [α-^{32}P]UMP was measured by scintillation counting. The transcription efficiencies of *trn*M2/fusion constructs relative to the wild-type *trn*M2 gene and 514 are presented in Table I. **Lane 1:** pdX11 plasmid DNA. **Lane 2:** control reaction without plasmid DNA. **Lane 3:** *trn*M2 deletion mutant 51. **Lane 4:** *trn*M2 construct 514. **Lane 5:** 51-*rbc*L fusion construct. **Lane 6:** 51-*psb*A fusion construct. **Lane 7:** 51-*atp*B fusion construct. The lower and higher mol. wt. transcripts represent unmodified and pseudouridylated tRNA$^{Met}_2$ transcripts, respectively (Greenberg *et al.*, 1984). **(B)** Kinetic analysis of tRNA$^{Met}_2$ transcription from *trn*M2/promoter fusion constructs. The different *trn*M2/promoter fusion constructs were compared for their transcription kinetics with the *trn*M2 wild-type gene and 514. The tRNA$^{Met}_2$ transcript was synthesized in the chloroplast extract for various times, and transcription was stopped with proteinase K/SDS. The tRNA$^{Met}_2$ transcription products were separated by electrophoresis on 10% polyacrylamide-50% urea gels and incorporation of [α-^{32}P]UMP was measured by scintillation counting of the excised tRNA bands.

Table I. Relative transcription efficiencies of *trn*M2 promoter fusion constructs

Templates	Transcription efficiency	
	(% of wild-type)[a]	(% of 514)[b]
51	4.8	–
514	67	–
51-*rbc*L	53	80
51-*atp*B	40	57
51-*psb*A	88	133
516	79	122 (75)[c]
518	16	22 (15)[c]
51/*psb*A-*trn*M2	112	151
51/*trn*M2-*psb*A	52	70
51/*psb*A-*rbc*L	73	98
51/*rbc*L-*psb*A	29	38.5

[a]Percent values reflect the incorporation of [α-^{32}P]UMP into mature tRNA$^{Met}_2$ transcription products as determined by scintillation counting of the excised RNA bands. The mature tRNA$^{Met}_2$ transcription products from the 290 bp *trn*M2 *Sau*3A-*Xba*I DNA restriction fragment in pdX11 were used as wild-type controls. Incorporation of [α-^{32}P]UMP into the control transcript was typically 0.8 – 1.2 c.p.m. × 10^{-4} with 10 μCi [α-^{32}P]UTP in the transcription reaction. The percent values were calculated from mean values of three or more transcription reactions of different experiments.
[b]The tRNA$^{Met}_2$ transcription products from the *trn*M2 514 promoter construction (Gruissem and Zurawski, 1985b; see Figure 1) were used as a control to quantitate transcription products from *trn*M2/promoter fusion constructs.
[c]Relative transcription efficiencies for *trn*M2-*psb*A promoter fusion constructs with single base mutations in the *psb*A promoter regions were calculated using the fusion construct 51-*psb*A (Figure 2) as a control.

51 basal level (Table I). While 51-*atp*B showed the lowest transcriptional activity (57% efficiency), 51-*psb*A was a template significantly better (133% efficiency) than the 514 construct. 51-*rbc*L directed transcription of tRNA$^{Met}_2$ with an ~30% higher efficiency than 51-*atp*B. The 'parental' *trn*M2 514 construct itself is slightly less efficient than the wild-type *trn*M2 sequence (Gruissem and Zurawski, 1985b and Table I), and therefore we have compared the rates of tRNA$^{Met}_2$ accumulation for the various constructs with 514. Figure 3B shows that variations between the constructs in the level of expression of tRNA$^{Met}_2$ reflects differences in the rate of accumulation of the tRNA. These rate differences are reproducible, linear over the time period, and are independent of DNA concentrations at saturating levels (see legend to Figure 3A). The mature tRNA$^{Met}_2$ product is stable in the extract for at least 1 h (data not shown). Furthermore, since our previous experiments with *trn*M2 confirmed that changes upstream of base pair −28 have no effect on tRNA$^{Met}_2$ processing (Gruissem *et al.*, 1983b), the differences observed must reflect differences in transcription efficiency.

These results show that ~40-bp regions proximal to, and including the transcriptional start sites of *rbc*L, *atp*B and *psb*A, encode sufficient information to functionally replace an equivalent region from *trn*M2. The different promoter constructs have distinct strengths as judged by the rate of tRNA$^{Met}_2$ accumulation in the *in vitro* extract. We will comment below on the possible physiological significance of the observed variations in promoter strength. It is also possible that DNA sequences from the intergenic regions 5′ distal to the DNA fragments used in the heterologous constructs are required in addition for the efficient recognition and/or control of promoter elements.

Promoter-down mutants in psbA

A direct test of the significance of the conserved sequence ele-

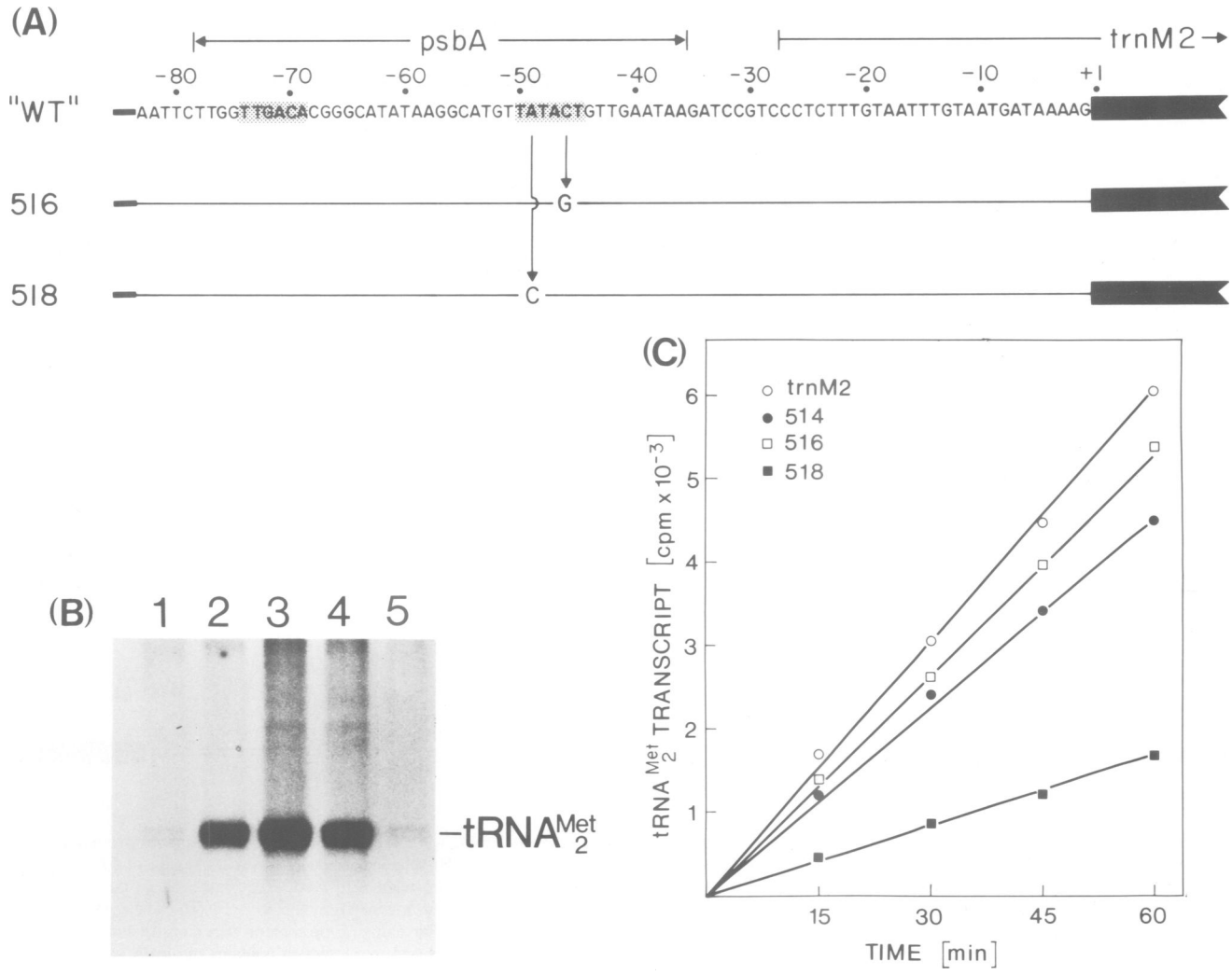

Fig. 4. Construction and *in vitro* transcription of *psb*A promoter mutants. **(A)** Single point mutations were introduced into the putative Pribnow box for *psb*A (base pairs −5 to −10 relative to the *psb*A transcription start site) by substitution of single base pairs in the complementary synthetic oligonucleotides at positions −6 (C to G, 516) and −9 (A to C, 518). The mutant oligonucleotides were subsequently fused to *trn*M2 mutant 51 as described in Materials and methods. **(B)** *In vitro* transcription of the mutant templates in the chloroplast extract was performed with form I DNA with equal copy numbers for each template DNA. Details of the transcription procedure and incubation conditions are described in Figure 3 and Materials and methods. **Lane 1:** *trn*M2 deletion mutant 51. **Lane 2:** *trn*M2 construct 514. **Lane 3:** 51-*psb*A promoter fusion construct. **Lane 4:** *psb*A promoter mutant 516. **Lane 5:** *psb*A promoter mutant 518. **(C)** Kinetic analysis of $tRNA_2^{Met}$ transcription from *psb*A promoter mutant constructs. 516 and 518 were compared for their transcription kinetics with the *trn*M2 wild-type gene and 514. Details of the analysis procedure are described in Figure 3.

ments within chloroplast promoters is to study the effect of nucleotide substitutions within those elements on promoter function. Our previous data have indicated that single base changes in the ctp1 sequence element of *trn*M2 can alter promoter function (Gruissem and Zurawski, 1985b). As a further test, we synthesized and cloned two mutant forms of the *psb*A promoter region in the *trn*M2 51 construct (Figure 4A). The *in vitro* transcription analysis shows that both mutants are transcribed, but their transcription efficiency is significantly reduced (Figure 4B). Mutant 516 changes the *psb*A sequence element TATACT to TATAGT. In *E. coli* promoters, the frequency distributions of residues at the −6 positions are 51% A, 19.5% C, 17% T and 12.5% G (Hawley and McClure, 1983). Thus, our finding that mutant 516 has a slightly reduced transcription rate (133% versus 122%, Table I) compared with the parental *psb*A-*trn*M2 construct is consistent with a prokaryotic model (i.e., tolerance for a G at the −6 position) for chloroplast promoter function. The second mutant, 518, changes the sequence element TATACT to TCTACT. A cytosine (C) residue is never found at this position in *E. coli* promoters (Hawley and McClure, 1983). Figure 4B and C shows that this mutational change results in drastic reduction (5.5-fold) in the rate of *psb*A-directed $tRNA_2^{Met}$ accumulation. Similar promoter-down phenotypes are observed when, for example, analogous A to C changes are introduced in the '−10' sequences of the *E. coli lac* and λ*PRE* promoters. These results confirm that the TATACT sequence element in the *psb*A promoter contains sequence information critical to promoter function and suggests that the analogous regions in the other chloroplast promoters that we have examined may carry comparable information.

Chloroplast promoter sequence elements are interchangeable

As described above, the promoter regions of *trn*M2, *rbc*L, *atp*B and *psb*A all have clearly identifiable sequence elements with homology to each other and to the canonical prokaryotic sequence elements. All three genes share the sequence 5′ TTG with ctp1 and the prokaryotic '−35' consensus sequence, but the immediate surrounding DNA sequences are not highly conserved. In the ctp2 region and the '−10', changes appear to be less frequent

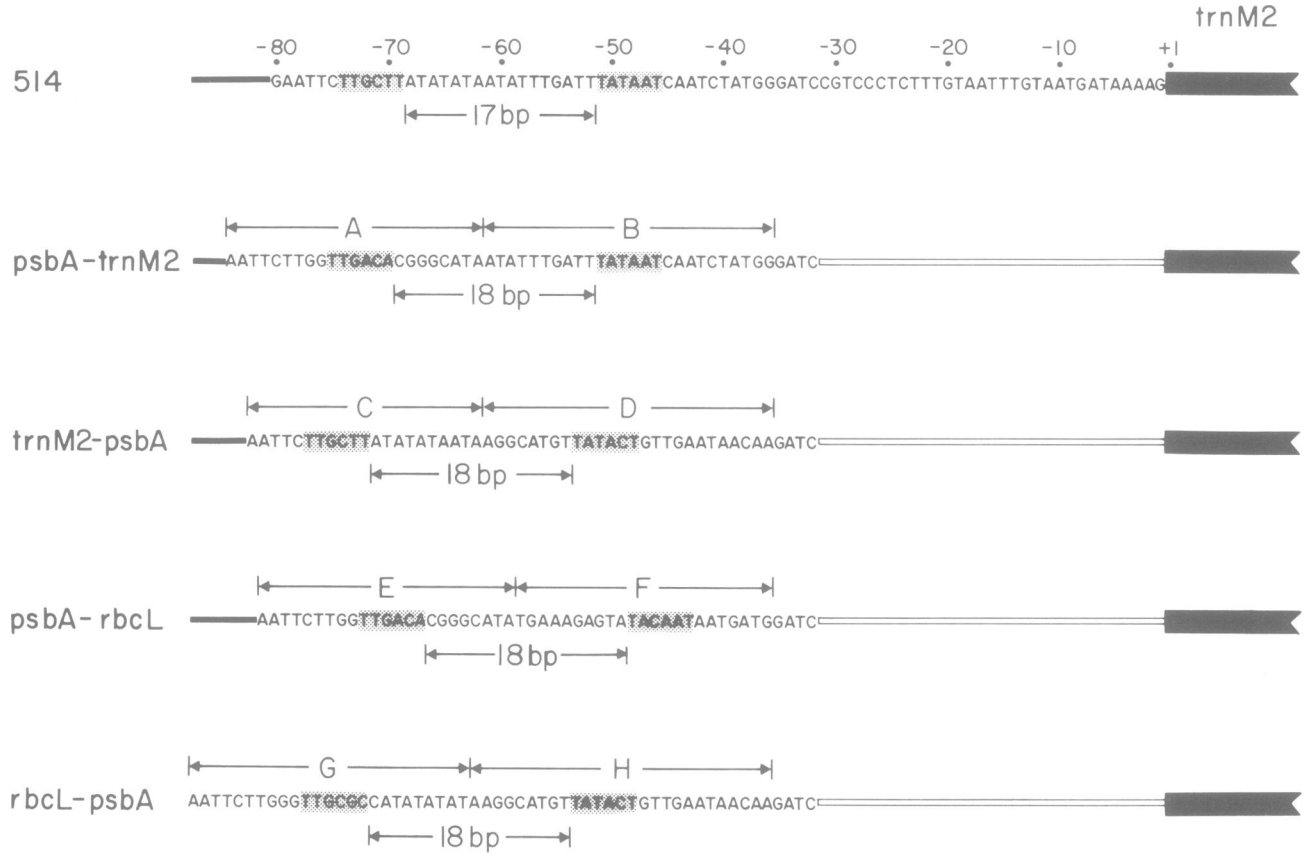

Fig. 5. Construction of chimeric promoter regions. Promoter elements from *trn*M2, *psb*A and *rbc*L were exchanged to construct chimeric promoter regions. In 51/*psb*A-*trn*M2 base pairs −21 to −38 **(A)** from *psb*A were fused to the *trn*M2 ctp2 sequence **(B)** retained in the 5′ upstream region of 514 to base pair −61. The spacing between the conserved regions has increased by 1 bp relative to 514, but is constant relative to *psb*A (see Figure 1). In the construct 51/*trn*M2-*psb*A the 514 upstream region from −35 to −61 was replaced by DNA sequences of the *psb*A gene from +5 to −18 **(D)**. The *trn*M2 ctp1 sequence is retained in this construct **(C)**, but the spacing was increased by 1 bp relative to 514. Similar chimeric promoters were constructed from promoter elements in the *psb*A and *rbc*L 5′-flanking regions. In 51/*psb*A-*rbc*L, a synthetic oligonucleotide was fused to 51, which contains the *psb*A sequence from −21 to −38 **(E)** and the *rbc*L sequence from +3 to −20 **(F)**. The chimeric promoter in 51/*rbc*L-*psb*A is composed of *rbc*L sequences from −20 to −39 **(G)** and *psb*A sequences from +5 to −19 **(H)**. Both constructs retain the original spacing (18 bp) between the conserved regions, which are indicated by sequences in the shaded boxes.

and are usually confined to a single base substitution (Figure 1). It is possible that these changes could account for the observed differences in promoter strength, and consequently transcription efficiency, for *rbc*L, *atp*B, *psb*A and *trn*M2. In *E. coli* promoters, '−35' and '−10' sequence elements, although generally recognizable, vary in specific sequence in ways that account for the strength and sometimes the regulation of the individual promoter. Experiments in the *E. coli* system have shown that it is possible to shuffle '−35' and '−10' regions between promoters and still maintain promoter function, albeit with differing regulatory and kinetic properties. These results reflect the non-specific spacer role of the region between the '−35' and the '−10' sequences. In previous experiments we confirmed that base substitutions in the comparable region of the spinach chloroplast *trn*M2 gene has only little effect on the *in vitro* transcription efficiency. To test if the identifiable sequence elements in the chloroplast promoters that we have characterized are themselves interchangeable, we constructed and cloned synthetic hybrid promoters between *psb*A and *trn*M2, and *psb*A and *rbc*L, and measured their ability to direct the synthesis of $tRNA_2^{Met}$ in the *trn*M2 51 tester system. Figure 5 shows the sequences of the four hybrid promoters tested. The spacing between the identified sequence elements was maintained at 18 bp.

Figure 6A shows that the four hybrid promoters are capable of directing the synthesis of $tRNA_2^{Met}$, although their rates of synthesis vary significantly relative to the parental promoter constructs (Figure 6B and Table I). Of the four hybrid promoters, *psb*A-*trn*M2 directs the highest rate of synthesis, and is in fact 20% more active in the transcription system than the *psb*A construct (Table I). It is interesting to note that the *psb*A-*trn*M2 promoter has the two sequence elements TTGACA and TATAAT that are identical to the canonical prokaryotic '−35' and '−10' sequence elements, respectively (Figure 5). Thus, we conclude that the identified promoter sequence elements for *trn*M2, *rbc*L, *psb*A, and, most likely, *atp*B, represent common elements which can be effectively replaced between genes.

Although sequences surrounding these chloroplast promoter sequence elements may also play a role in defining promoter strength, a preliminary model of chloroplast transcriptional initiation can be presented. First, a two part promoter analogous to the *E. coli* RNA polymerase promoters is utilized in the expression of chloroplast mRNA genes. Next, ctp1 sequences can be ordered TTGACA > TTGCTT > TTGCGC with respect to their intrinsic strengths. We note that this is simplistic, in that it does not address the relative and presumably subtle contribution of each region to the activity of the promoter. However, in each combination of ctp1 sequences with any ctp2 sequences, the order presented above for promoter efficiency is maintained. There are

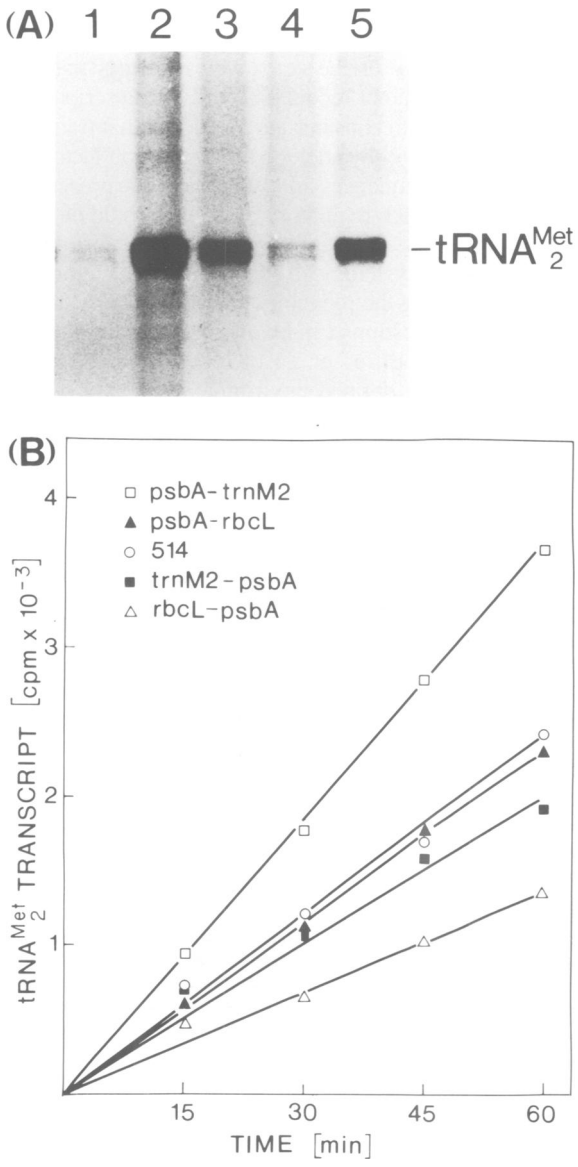

Fig. 6. Direction of *trn*M2 transcription by chimeric promoter regions. **(A)** The strength of the chimeric promoter constructs described in Figure 5 was estimated by their efficiency to direct transcription of $tRNA_2^{Met}$ in the chloroplast extract. Details of the transcription procedure and quantititation of $tRNA_2^{Met}$ products are described in Figure 3. **Lane 1:** *trn*M2 deletion mutant 51. **Lane 2:** 51*psb*A-*trn*M2 (A/B). **Lane 3:** 51/*trn*M2-*psb*A (C/D). **Lane 4:** 51/*rbc*L-*psb*A (G/H). **Lane 5:** 51/*psb*A-*rbc*L (E/F). The transcription efficiencies for the *trn*M2/chimeric promoter templates are given in Table I. **(B)** Kinetic analysis of the *trn*M2/promoter constructs described in Figure 5. Transcription kinetics of these templates were compared with *trn*M2 construct 514. Details of the analysis procedure are described in Figure 3.

insufficient data to order the ctp2 sequences, other than the observation that the combination with complete homology to the canonical promoter sequences apparently are most efficient for transcription *in vitro*.

Discussion

We have used a chloroplast transcription extract and a *trn*M2 promoter deletion mutant to develop a general and rapid assay system for plastid promoter regions and the mutational analysis of their DNA sequences. The present work shows that DNA sequences from 5′ upstream regions of plastid genes are capable of directing the expression of the heterologous *trn*M2 gene *in vitro*. The promoter regions we utilized had previously been well characterized with respect to at least the site of transcription initiation *in vivo*. However, we feel that the methodology presented here can be utilized as a test for any suspected plastid promoter regions. Previous studies, relying on comparison of the sequences of putative chloroplast promoter regions and on the ability of prokaryotic RNA polymerase to recognise with fidelity certain chloroplast promoters (Tohdoh *et al.*, 1981; Zech *et al.*, 1981; Gatenby *et al.*, 1981; Hanley-Bowdoin *et al.*, 1985a; Shinozaki and Sugiura, 1982; Erion *et al.*, 1983; Dzelzkalns *et al.*, 1984) have led to the notion that chloroplast RNA polymerase is a direct analogue of prokaryotic RNA polymerase. Our results extend this notion in several ways.

First, in both the prokaryotic and the chloroplast systems, limited regions of ~40 bp proximal to, and including, the transcription start site carry sufficient information for promoter function. For example, the comparison of sequences in the intergenic region between *rbc*L and *atp*B from spinach, tobacco, maize and pea uncovers extensive sequence homology in the immediate 5′-flanking regions (+1 to −40) of the two genes (*rbc*L: 85%, *atp*B: 85%, except for maize, where the homology is only 42.5%). The DNA sequences immediately proximal to these conserved regions are randomized between species (Mullet *et al.*, 1985). Therefore, if common promoter elements are required for transcription initiation of these genes in higher plants, they are most likely contained in the 40-bp regions 5′ distal to the transcription start sites.

Second, sequence elements that are analogous to the prokaryotic '−35' and '−10' regions are recognizable in many chloroplast promoters. As we have shown above, DNA sequences upstream from the '−180' and '−455' termini of *rbc*L and *atp*B, respectively, are characterized by the presence of promoter elements that are similar or identical with the prokaryotic consensus promoter. In addition, the lack of $tRNA_2^{Met}$ transcription from templates in which the promoter region of the *trn*M2 gene had been replaced with random DNA sequences supports our notion that the synthetic DNA fragments contain highly specific sequences required for transcription of *rbc*L, *atp*B and *psb*A. We note, however, that a putative *atp*B ctp2 sequence diverges both in nucleotide sequence and arrangement from the corresponding sequence of the *rbc*L gene. A direct test for the significance of these sequences in both systems is the mutagenic alteration of these regions. Previous data (Gruissem and Zurawski, 1985b) and the analysis described here confirm that single base changes in these two regions can dramatically alter chloroplast promoter function.

Third, the spacing between the '−35' and the '−10' sequence elements is critical for promoter function in the prokaryotic system (17 ± 1 bp), while the spacer sequence itself can generally be altered without altering the function of the promoter (excluding superimposed regulatory sequences). Characterizations of the effect of spacer length on the activity of *E. coli* RNA polymerase at the *lac* p^s promoter *in vitro*, the *trp-lac*UV5 promoter *in vitro* and *in vivo* and the *tet* gene promoter *in vivo* have convincingly demonstrated that consensus promoter sequences direct transcription most efficiently (Stefano and Gralla, 1982; Aoyama *et al.*, 1983; Mulligan *et al.*, 1985; Brosius *et al.*, 1985). Our experiments with the spinach chloroplast *trn*M2 promoter (Gruissem and Zurawski, 1985b) and our sequence element shuffling experiment presented here also follow this rule.

Fourth, in the prokaryotic promoter, the sequences of the '−35' and '−10' regions, although following a general pattern,

are variable. This variation accounts for the vast range of promoter strengths, affecting such parameters as polymerase binding and open complex formation (McClure, 1980; Hawley and McClure, 1980b, 1982; Simons *et al.*, 1983). The nature of the interaction of the '−35' and '−10' regions in the prokaryotic system is such that these regions can often be interchanged between promoters (while maintaining spacing), yielding functional promoters with new and distinct kinetic properties (e.g., the *E. coli tac* promoter has the '−35' region of the *trp* operon and the '−10' region of the *lac* operon). Our experiments show that the chloroplast analogues of the '−35' and '−10' regions are also interchangeable and yield functional promoters with a greater range of apparent strengths than the natural promoter themselves.

Finally, the prokaryotic promoters that are considered most powerful are often most closely related to the canonical sequence (e.g., the *tac* promoter). It is of interest that the hybrid chloroplast promoter 51/*psb*A-*trn*M2, which contains 5′ upstream sequences from *psb*A and *trn*M2 that have absolute homology to the canonical '−35' and '−10' regions, yields the greatest rate in the *in vitro* transcription system.

Despite the analogy between prokaryotic and chloroplast promoters, however, we feel that a number of questions remain to be elucidated. For example, each transcription system has a specificity as yet not understood. Although chloroplast promoter regions share significant functional and sequence homology with prokaryotic promoters, the chloroplast transcription extract fails to recognize the *lac*UV5 promoter (Gruissem *et al.*, 1983b) or the *Klebsiella pneumoniae nifH* promoter (data not shown). Also, the precise nature of the differing strengths of natural and hybrid chloroplast promoters is at present unknown. For example, our mutational and shuffling experiments lack the resolution to exclude the possibility that residues immediately adjacent to the conserved sequence elements affect promoter activity. It has been suggested for the mustard chloroplast *psb*A promoter that a sequence in the spacer between the '−35' and '−10' regions with homology to the nuclear TATAA promoter element can influence the transcription of this gene (Link, 1984). Ultimately this question can best be answered with a saturating mutational analysis. The analysis of mutational effects described above addresses only the rate of accumulation of the RNA product. It will be of further interest to determine if mutations exist that have differential effects on, for example, promoter-RNA polymerase binding or rate of initiation by pre-bound RNA polymerase.

We do not know whether the difference in transcription efficiency from the *rbc*L, *atp*B and *psb*A promoters observed *in vitro* is reflected *in vivo*. Preliminary data, however, indicate that the *in vivo* transcription modes may be conserved *in vitro*. In tobacco the steady-state levels of *rbc*L mRNA are greater by several fold than *atp*B mRNA (Shinozaki *et al.*, 1983). Similar results have been obtained from *in vitro* transcription experiments with a template containing the intact *rbc*L/*atp*B intergenic region and transcription initiation sites for these genes (L.Hanley-Bowdin, E.M.Orozco and N.H.Chua, personal communication). These observations, coupled with the low abundance of *atp*B mRNAs relative to the *rbc*L transcripts (Mullet *et al.*, 1985), suggest that the distinct transcription efficiencies for the *rbc*L and *atp*B promoters could be a function of their intrinsic properties. A complete answer to this question, however, requires careful comparative estimates of chloroplast mRNA levels and half-lives that are not yet available.

Many chloroplast promoters are subject to environmental regulation (Bedbrook *et al.*, 1978; Smith and Ellis, 1981; Nelson *et al.*, 1984; Rodermel and Bogorad, 1985). Our expectation is that some of these modes of regulation will operate at the level of RNA polymerase-promoter recognition. We note that DNA sequences in the vicinity of the conserved elements may play a significant role in promoter recognition and/or transcription regulation. Also, we have to consider the possibility that transcription may be subject to control by additional transcription factors which can recognize such sequences and which are not present in our chloroplast extract. Such regulatory molecules could be required during different stages of plastid development and differentiation or under different physiological conditions (i.e., light quality/intensity). Considering the reliability of the chloroplast transcription system, these questions can be addressed in the future with extracts isolated from different plastid types or chloroplast from plants growing under defined environmental conditions.

Materials and methods

Reagents

Ribonucleotides and deoxyribonucleotides were from Pharmacia/P-L Biochemicals, Inc. Protected deoxynucleotides for oligonucleotide synthesis were from Applied Biosystems, Inc. Nucleic acids were deproteinized by treatment with proteinase K and phenol-chloroform-isoamylalcohol (25:25:1). Acrylamide sequencing gels were prepared with electrophoresis-purity acrylamide and N,N′-methylenebisacrylamide from Bethesda Research Laboratories. Electrophoresis-grade agarose, enzyme-grade urea and ammonium sulfate, biological-grade cesium chloride and nucleic acid-grade formamide were also from BRL, Inc. Formamide was deionized for 1 h at 20°C with AG 501-X8(D) analytical grade mixed bed resin (BioRad Laboratories) and stored at −20°C. Pre-swollen DE-52 resin was from Whatman Chemical Separation Ltd.

Enzymes

Restriction endonuclease enzymes, T4 polynucleotide kinase, T4 DNA ligase and DNA polymerase I (large fragment) were from BRL, Inc. Calf intestine phosphatase was from Boehringer Mannheim.

Plant growth and chloroplast isolation

Spinacea oleracea seeds (Marathon hybrid) were purchased from Asgrow Seed Company. Seedlings were grown to a length of 1 in. in sterilized soil and then transferred to tanks and grown hydroponically in one half strength Hoagland's solution under greenhouse conditions (Gruissem and Zurawski, 1985b). Leaves 5 − 10 cm in length were used for the isolation of intact chloroplasts (Gruissem, 1984). Intact chloroplasts were isolated after centrifugation through Percoll gradients as described (Price and Reardon, 1982).

Synthesis of oligonucleotides

Oligonucleotides were synthesized using the automated Applied Biosystems synthesizer. Deprotected products were size fractionated on sequencing gels, eluted by soaking and further purified by DEAE-cellulose chromatography.

Construction of trnM2/promoter fusions

A 290-bp *Sau*3A-*Xba*I fragment containing the *trn*M2 locus and 98 bp of 5′ upstream DNA was cloned into the *Bam*HI-*Xba*I restriction sites of pdX11. pdX11 is a derivative of pUC8 with additional *Xba*I and *Bgl*II restriction enzyme sites between the *Pst*I and *Hind*III restriction enzyme sites in the polylinker region (M.Benedik, personal communication). The construction of *Bal*31 deletion mutants of the *trn*M2 5′ upstream region in this vector has been described (Gruissem and Zurawski, 1985b). Deletion mutant 51 (deletion endpoint at base pair −28) was used to construct the promoter fusion templates. The synthetic oligonucleotides were designed with 5′ *Eco*RI and 3′ *Bam*HI compatible ends. The *Bam*HI restriction enzyme site in the deletion mutant 51 had been restored by cloning the *Bal*31-treated and *Xba*I-cut DNA fragments into the *Hinc*II/*Xba*I restriction enzyme sites of pdX11. Deletion mutant plasmid DNA was digested with *Eco*RI and *Bam*HI and subsequently incubated with calf intestine phosphatase enzyme for 30 min at 37°C. The phosphatase enzyme was inactivated by incubation of the reaction mixture at 65°C for 10 min, followed by deproteinization with phenol-chloroform-isoamylalcohol. Approximately 120 − 150 pmol of synthetic oligonucleotides were phosphorylated on the 5′-OH ends in 15 μl reactions containing ATP and T4 polynucleotide kinase. 10 − 20 pmol of the complementary synthetic nucleotides were mixed with the restriction enzyme-digested *trn*M2 deletion mutant plasmid DNAs. The mixture was briefly heated to 65°C, cooled to 25°C and ligated with 0.1 unit T4 DNA ligase for 4 h in 20 μl reactions. Competent JM103 cells were transformed with 10 μl of the reaction mixture (Messing, 1983), and ampicillin-resistant colonies were screened for the correct single insertion of the synthetic DNA fragments by digestion of plasmid DNA from alkaline mini-preparations with *Eco*RI and *Xba*I. The constructs were verified by sequencing the supercoiled

plasmid DNAs. Mini-prep DNAs were denatured with 0.2 N sodium hydroxide, precipitated with ethanol and the denatured double-stranded DNA was sequenced using the reverse sequencing primer, dideoxynucleotides and the large fragment of DNA polymerase I (Sanger *et al.*, 1977).

Isolation of plasmid DNA

Plasmid DNAs were isolated by a modified cleared lysate procdure (Clewell, 1972) or the alkaline-SDS method (Birnboim and Doly, 1979). The crude plasmid DNA fractions were incubated with RNase and proteinase K prior to centrifugation. Supercoiled DNA was purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients for 8 h in the Sorvall TV865 vertical rotor. Purified plasmid DNAs were routinely analyzed by agarose gel electrophoresis and were typically >80% form I DNA.

Chloroplast transcription extract

The chloroplast extract was prepared according to a described procedure (Gruissem *et al.*, 1983b, 1986; Gruissem, 1984). Briefly, isolated intact chloroplasts were lysed in a hypotonic buffer and stromal proteins were extracted with 0.5 M ammonium sulfate. After removal of the membrane material, including most of a tightly DNA-bound RNA polymerase (transcriptionally active chromosome, TAC; Gruissem *et al.*, 1983b), by high speed centrifugation, remaining nucleic acid/protein complexes were removed by DEAE column chromatography of the supernatant fraction. The proteins from the DEAE column fraction were precipitated with ammonium sulfate. After resuspension of the protein pellet and dialysis, the extract was used for transcription experiments.

In vitro transcription reactions

Plasmid DNAs (predominately form I DNA; 60 μg/ml) were incubated under standard conditions as described elsewhere (Gruissem *et al.*, 1986). Radioactively labeled *in vitro* RNA transcription products were separated on 10% polyacrylamide-50% urea gels, and the transcription efficiency was calculated by measuring the incorporation of [α-^{32}P]UMP into mature tRNA transcription products.

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